Vol. 12 May 1936 No. 5

Bulletin of the Agricultural Chemical Society of Japan.

TRANSACTIONS

Biochemistry of Filamentous Fungi. IV.

Mycelial Constituents of Oospora sulphurea-ochracea. Part I.

By Hidejiro NISHIKAWA.

(Tottori Agricultural College, Japan.)

(Received March 20, 1936.)

Some time ago the writer reported the isolation of crystalline constituents of Oospora aurantia⁽¹⁾, the chief and usually occurring compound being a yellow colouring matter, aurantin. As the genus Oospora includes a number of coloured species, it was anticipated to find aurantin or related substances in the mycelium of these species and thus to prove parallelism between biochemical and morphological characteristics. Four available coloured specimens: Oospora crustacea, O. fulva, O. sulphurea-ochracea, and O. sulphurella were purchased for this purpose from the Centraalbureau voor Schimmelcultures at Baarn and cultural and chemical experiments were started.

Raisin decoction medium which was so far the best one for the isolation of crystalline constituents from the mycelium of O. aurantia has proved to be unsuitable for the Oosporae above mentioned. There was no medium which was equally favourable for all these four species. They differ one another in their nutritional requirements. O. sulphurea-ochracea grow very well in koji wort which is also fairly good for O. fulva. While the isolation of aurantin or related pigments from these Oosporae is yet unsuccessful, several crystalline substances were found to be present in the mycelium of O. sulphurea-ochracea and it was attempted to elucidate their chemical nature. The mode of isolation and some properties of them are described below.

EXPERIMENTAL

Cultural Conditions and Growth.

Koji wort was the sole medium tried for the cultivation of this mould. When prepared according to the routine process koji wort has the concentration of about 12° Balling and pH in the neighbourhood of 5.6. Conical flasks containing koji wort of various concentrations from 6° up to 12° Bllg. were prepared and the growth therein was examined. The yields were nearly equal in each concentration, but in 6° Bllg. colonies, though large in number, are small and thin while in 12° Bllg. thick, scattered colonies developed. In the neighbourhood of 8° Bllg. colonies unite themselves into a mat of moderate thickness which is convenient for cropping and handling. As for pH value 3 to 7 were tested. In pH 3 there was no growth. In flasks from 4 to 7 the yield of dried mycelium showed no marked difference, slightly better in the neighbourhood of 6. The appearance of mycelial felt, however, showed remarkable dissimilarity along with pH. In pH 4 the felt was thin and shrunk while in pH 5 to 7 the felt looked white covered with colourless hyphae and in pH 7, moreover, the reverse side of the felt coloured dark brown. From these experimental results koji wort diluted to 8° Bllg. was employed as the mass culture medium throughout the investigation.

Mass Production of the Mycelium.

Koji wort of 8° Balling was distributed, in 250 ml quantities, into a number of 2 litre conical flasks and sterilised for three successive days. Small pieces of mycelium cut from colonies on koji wort agar slope were inoculated into each of the flasks and incubation was continued for about thirty days at 20°. Cropped sheets of mycelium were pressed, dried in vacuo over sulphuric acid and pulverised. The average yield of dried mycelium was about 1.2 g per 100 ml of culture solution.

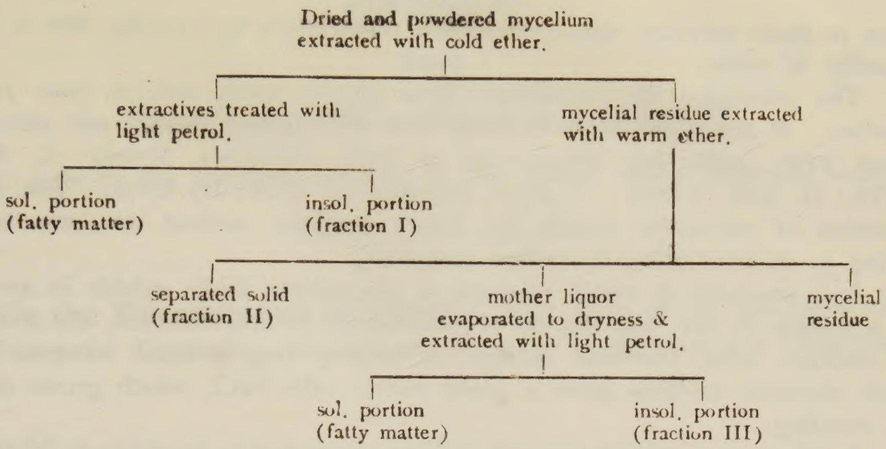
Extraction and Isolation of Crystallin compounds.

Powdered mycelium was extracted first with cold ether renewing the solvent several times until the colour of the last lot of solvent grew pale yellow. The material was then subjected to warm ether extraction using a large Soxhlet apparatus. During a few days' extraction yellowish crystalline mass gradually deposited on the bottom of the flask. It takes usually a week or longer to extract nearly all ether-soluble matter from the mycelium. The yield of ether extractives was generally 4 to 6 per cent. of dried mycelium. Mycelial residue from warm ether extraction was preserved for further investigation.

Ether was driven off from the cold ether extract and the residue was treated with light petroleum to remove fatty matter. From the warm ether extract too, after filtering off the separated solid, the solvent was distilled off and the residual extractives were treated with light petroleum. The ether soluble matter was thus fractionated as tabulated below.

Fraction I.

Dissolved in acetone and treated with animal charcoal. The filtrate was poured into a large amount of water. A brown-tinted precipitate which separated out was filtered, dried on porous porcelain and digested with a



small quantity of warm ethyl alcohol. Nearly colourless crystals which remained undissolved were then recrystallised from hot alcohol. Colourless needle crystals were obtained, m.p. 214° (A). (Analysis. Found: C, 58.59, 58.47 ; H, 4.82, 4.91% ; Mol. wt. by Rast's camphor method. 334.6. $C_{17}H_{16}O_8$ requires: C, 58.6 ; H, 4.6% ; Mol. wt. 348).

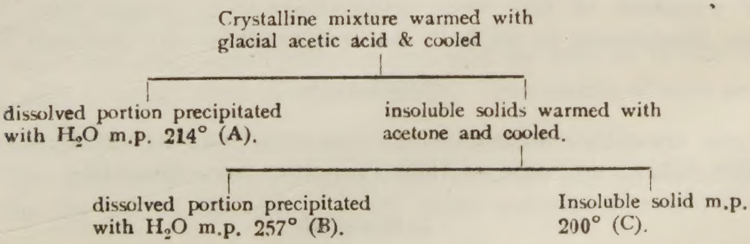
This substance (A) is insoluble in water or benzene, moderately soluble in acetic ester, readily soluble in acetone. In hot methyl or ethyl alcohol it is soluble and separates out slowly on cooling. Its solubility in ether is slight, somewhat better when warmed.

It is soluble in NaOH and $NaHCO_3$ solution and reprecipitated unchanged by acid. Its alcoholic solution gives a violet colour with $FeCl_3$.

In one case from this fraction another crystalline substance of m.p. 147° was separated which has not been analised yet.

Fraction II.

Decolorised with animal charcoal in hot acetone and poured into a large amount of water. A pale yellow coloured precipitate separated out. This precipitate was in some cases composed of a single substance of m.p. 257° but usually a mixture of compounds at least three, the predominant one being that of 257°. They were roughly separated by treating with glacial acetic acid and acetone in succession as tabulated below.


Crystalline mixture warmed with
glacial acetic acid & cooled
dissolved portion precipitated
with H₂O m.p. 214° (A).
insoluble solids warmed with
acetone and cooled.
dissolved portion precipitated
with H₂O m.p. 257° (B).
Insoluble solid m.p.
200° (C).

In a few cases from the fraction soluble in glacial acetic acid another substance of m.p. 190°, (D), was obtained instead of (A). The soluble portions in these solvents were precipitated respectively by pouring into a large quantity of water.

The substance (B) crystallizes from glacial acetic acid in pale yellow needles. It turns yellow on heating over 230°, grows darker and sinters at about 240°, melts and decomposes at 257°. (Analysis. Found: C, 61.42, 61.76; H, 5.31, 5.29%. $C_{10}H_{10}O_4$ requires: C, 61.8; H, 5.1%. The determination of molecular weight by Rast's camphor method was unsuccessful owing to its insolubility in molten camphor.)

It is insoluble in water, benzene or chloroform, fairly soluble in acetone. It is soluble in hot, and less so in cold methyl or ethyl alcohol, but it seems to undergo some chemical change by heating long in these solvents Its ethyl alcoholic solution gives a green colour with $FeCl_3$ which grows darker on standing.

It dissolves in NaOH in bright yellow colour but is insoluble in $NaHCO_3$.

The substance (C) crystallizes from ethyl alcohol in rhombic block crystals. It melts at 200° with effervescence. (Analysis. Found: C, 58.83, 58.68; H, 4.94, 4.95%; Mol. wt. by Rasts camphor method. 334.7. $C_{17}H_{16}O_8$ requires: C, 58.6; H, 4.6%; Mol. wt. 348.) Probably an isomer of (A).

It is insoluble in H_2O or benzene, hardly soluble in acetone or chloroform, sparingly soluble in ether, fairly soluble in hot methyl or ethyl alcohol. Its alcoholic solution given no colour with $FeCl_3$. It is soluble both in NaOH and $NaHCO_3$.

The substance (D) crystallizes from ethyl alcohol in stumpy prisms or blocks. It melts at 190°. (Analysis. Found: C, 59.51, 59.87; H, 5.17, 5.22%. $C_{18}H_{18}O_8$ requires: C, 59.7; H, 5.0%. Rast's camphor method gave no definite value.)

It is insoluble in water, benzene or chloroform, sparingly soluble in ether, moderately soluble in methyl or ethyl alcohol, readily soluble in acetone. Its alcoholic solution gives a violet colour with $FeCl_3$ which gradually fades away on standing. It is soluble in NaOH but insoluble in $NaHCO_3$.

The molecular formulae of these compounds are of provisional nature and should be confirmed or revised by further experiments.

Fraction III.

This fraction mainly consists of (A).

Further investigations are in progress.

The expenses of this work were defrayed by grants from the Nippon Gakujutsu Shinkôkwai to which I desire to express my sincerest thanks.

SUMMARY

Several crystalline substances are extracted from the mycelium of Oospora sulphurea-ochracea and some of their properties are described.

LITERATURE

(1) Proc. Imp. Acad., X, 414 (1934).

ABSTRACTS

from

TRANSACTIONS published in JAPANESE

(Pages refer to the Japanese originals of this volume unless otherwise noticed)

On the Regeneration of Fibroin. (III).—Fibroin sol. (pp. 343～346): By Takayoshi TANIZAKI. (Agr. Chemical Laboratory, Imperial University of Tokyo, Japan.)

Direct pH Determination of Soil under Its Natural State by Quinhydrone Method. II.—Description of a New Electrode and its Use. (pp. 347～355): By Arao ITANO and Yasuhiko TSUJI. (Ohara Agricultural Institute, Kurashiki, Japan.)

In the previous publication(1) the direct method as proposed by Verwell(2) was tested and the experimental results were presented. In the course of previous determinations, it was found that the electrode as adapted formerly was rather difficult to manipulate owing to the complexity of that part.

In this Paper, a new and convenient form of electrode which was devised by the senior author, is described together with the experimental results. The new electrode is built in one piece of ebonite which contains both the platinum and calomel electrodes. The saturated KCl calomel electrode is encased in an ebonite tube of which the end is pointed, and platinum wire is wound round near the pointed end and led up through the wall of ebonite casing. The connection between the calomel cell and platinum wire is made through an opening just above the platinum wire, which is plugged with a cork previously treated with KCl solution.

The electrode thus prepared can be pushed into the soil or other samples, after the platinum wire is smeared with quinhydrone paste, and the E. M. F. is determined by the potentiometer as usual.

The new electrode was tested in the field as well as in the laboratory, and found that it gives very accurate results. The merits of such an electrode may be as follows:

1) The pH values can be determined under the natural state, at any distance, for the soil in the field, or for other samples in semi-solid or solution.

2) The electrode is always ready to be used conveniently.

LITERATURE

(1) A. Itano and Y. Tsuji: Berichte d. Ohara Inst. f. landw. Forsch., VI, 587 (1935).
(2) H. J. Verwell: Soil Research, IV, 67 (1934).

Two New Yeasts.—Saccharomyces robustus nov. sp. and Saccharomyces praecisus nov. sp. (pp. 356～366): By Ryoji NAKAZAWA and Mituo SHIMO. (Department of Industry, Gavernment Research Institute, Taiwan, Japan.)

Studies on the Microorganisms in the Manufacture of "Miso".—Part I.(Vol. 10, No. 9, 983, 1934): By Masatoshi MOGI. (The Brewing Laboratory of Noda Shoyu Co. Ltd., Noda-machi, Chiba-ken, Japan.)

"Miso" or fermented soy-bean paste is a yellowish white or reddish brown uniform mass with a pleasant aromatic flavour and a peculiar salty taste. It forms an essential part of daily menu of the Japanese people and is almost as indispensable to them as rice. Its annual production in recent years is estimated more than twenty millions of yen.

EXPERIMENTAL

The samples used in this experiment attained to forty-eight, which were prepared by twenty-two chief "Miso" Manufacturing Factories of this country.

The names of the manufactures and the proportional quantities of the raw materials of the samples are given on the original report.

For the isolation of bacteria the plate cultures are made with neutral koji and broth agar. A small quantity of calcium carbonate are added to the former, to detect lactic and acetic acid bacteria. Besides bacteria, yeasts and moulds were also isolated and preserved for further research. Some anaerobic bacteria were also isolated by methods of Buchner, Burri, Botkin, Itano⁽¹⁾ and Ishimaru⁽²⁾.

Numbers of strains of Bacteria, yeasts and moulds isolated from "Miso".

Kinds of "Miso" (cipher)	Number of strains isolated		
	Bacteria	Yeast	Mould
h_1	11	2	1
h_2	21	4	1
e_1 e_3 e_4 e_6 f	24	2	4
i	8	2	2

v	4	5	2
c_1 c_3 c_4	29	2	3
u	2	1	1
a_1 a_2	7	4	
j	7	2	
s	8	1	1
w_1	7	1	1
g	2	2	1
x	4	1	1
y	4	1	1
z	4	1	2
n	8	2	1
m_1 m_2 m_3 m_5	9	4	2
k_2	5	1	1
k_1	3	1	1
l_2	2	1	1
l_1	1	1	1
b	2	1	2
g_1	6		
Total	178	42	30

The following investigations were made to confirm whether these bacteria were concerned in the manufacture of miso or not, using miso-soup, steamed soy-beans and neutral koji-water with 10% NaCl as the nutrient media.

(i) "Miso"-soup culture.

250 grs of the white miso and 1000 c.c. of distilled water is thoroughly mixed and filtered. The filtrate is sterilized in test-tubes according to the usual method. The test tubes thus prepared are inoculated with bacteria from the fresh agar stroke cultures and kept at 37°C for 2 days. The result of this experiment shows that most of them grow very well in this special medium.

(ii) Culture on steamed soy-bean.

Soy-bean is macerated in water for 12 hrs, and then autoclaved for 50 minutes at 15 to 20 pound per square inch, and then put into test-tubes, and sterilized. The bacteria are inoculated and incubated at 32°C for 4 days. All strains of the bacteria isolated, grow luxuriantly upon the above media.

(iii Culture on neutral koji-water with 10% NaCl.

The result of the cultivation on the neutral koji-water with 10% NaCl shows that most strains grow well on this medium, while ten out of 178 strains attain very poor growths.

From the results of the above three experiments, it is understood that almost all of the bacteria isolated from "Miso" are to some extent concerned in the "Miso" manufacture or the maturation during its storage.

The rates of the production of acid by above bacteria are also investigated using neutral koji-water (Blig. 7°). The details will be published later.

REFERENCES.

(1) A. Itano: J. Agr. Chem. Soc. Japan, 6, 996 (1930).
(2) Y. Ishimaru: "Jōzō-Gaku-Zasshi"., 8, 924 (1930).

Studies on the Microorganismus in the Manufacture of "Miso".—Part II. (pp. 367～377): By Masatoshi MOJI. (The Brewing Laboratory of Noda Shoyu Co. Ltd., Noda-machi, Chiba-ken, Japan.)

The following experiments were made with the aerobic bacteria⁽¹⁾ which were isolated from "Miso".

1. Diastatic and starch liquifying power.

Nutrient medium: peptone 1.0 g, $MgSO_4$ 0.5 g, NaCl 0.1 g, K_2HPO_4 0.1 g, starch 5.0 g and distilled water 100 c.c.

The starch paste of above constitution is equally divided into test-tubes and sterilized. They are inoculated and incubated at 30°C for 15 days. Liquifying power is determined by the estimation of heights of liquified portions. Diastatic power is determined by the estimation of sugar produced from starch, and by the iodine reaction.

2. Liquifying power of gelatine.

Nutrient medium: peptone 1.0 g, glucose 1.0 g, NaCl 0.5 g, gelatine 20% and neutral broth 100 c. c.

10 c.c. of the medium are sterilized in test-tubes of a similar dimention, inoculated and incubated at 16°C for 30 days. Liquifying power is determined by the height of liquified portion, and the modes of liquifaction are also observed. The result of this experiment shows that all the bacteria liquify gelatine.

3. Liquifying power of "Tofu".

"Tofu" or soy-bean curd, which is prepared by the coagulation of protein matters of soy-bean milk through the addition of Mg-salt, is used as a substratum for comparing the proteolytic activities of the bacteria. The Tofu is sterilized in test-tubes, inoculated with the bacteria and incubated subsequently at 30°C. After 40 days the height of its liquified portion are measured. The degrees of liquifaction are not always parallel to gelatine liquifying powers.

SUMMARY

(1) The author has studied the starch liquifying and diastatic power of

bacteria, isolated from miso, using three kinds of starch — rice, potato and soluble starch.

(2) The proteolytic activities of Bacteria against gelatin and Tofu have also been investigated.

(3) From these experiments, it is understood that sugar, dextrin and soluble decomposed products of protein in "Miso" may be produced by the action of bacteria as well as Koji moulds. Although the maturation or the decomposition of the protein and starch of the materials of "Miso" has hitherto been believed to be effected chiefly by the latter.

The author wishes to express his deep gratitude to Prof. Dr. T. Takahashi and Assit. Prof. Dr. K. Sakaguchi for their kind advices and encouragement throughout the work and also to the Director W. Mogi of this Laboratory. Thanks are also due to Mr. S. Yoshiike and Mr. S. Yoshida for their kind help.

LITERATUES

(1) M. Mogi: J. Agr. Chem. Soc. Japan., **10**, 983—996 (1934).

Studies on the Amylase of Yeast. VIII.—On the Enzym-chemical Properties. (5). The Effects of some Inorganic and Organic Substances on Saccharifying Power of the Yeast Amylase. (pp. 378～385): By Kazuki OHNO.

(Biochemical Laboratory of Taihoku Imperial University, Japan.)

Studies on the constituents of "Natto", the Fermented Soy-beans. Part I.—On the effect of dipicolinic acid produced in "Natto". (pp. 386～394): By Shigezō UDŌ. (Agricultural College of Morioka, Japan.)

The natto, or the fermented soy-beans, constitutes one of the many food-articles of some Japanese. When the well boiled soy-beans, to which Bacillus natto is introduced, are left to ferment at 40°C for about 20 hrs., a kind of viscid substance will be produced around each of the bean grains. These fermented beans with sticky coating on are no other than the so-called "natto".

Tyrosine, leucine, alanine, glutamic acid, phenylalanine, valine, ammonia, guanine, xanthine, Hypoxanthine, arginine, histidine and others have already been reported to occur in natto as its constituents. All of these are, however, the decomposed products of the soy-bean protein.

The present writer was, however, able to isolate pyridinedicarboxylic acid $(C_7H_5NO_4)$ from the ether extract of the viscous substance of natto. There are seven isomers in pyridinedicarboxylic acids. These are chinolinic acid, lutidinic acid, dipicolinic acid, isocinchomeronic acid, cinchomeronic acid, dinicotinic acid, and dipyridinetetracarboxylic acid, which is polymer. The acid the writer has obtained chrystalizes out from thick hot water solution in forms of prisms and plates, and in filiform from cold water solution. It is almost insoluble in ether unless sulfuric acid is presented in addition. It is soluble in methyl alcohol, ethyl alcohol and butanol, but not at all in chloroform, benzene and acetic acid. This acid shows orange red colour when the solution of ferrous sulphate is added. It decomposes at 245°C (uncorr.) When heated, it decomposes to become picolinic acid, pyridine and carbon dioxide. From these features this acid was determined to be dipicolinic acid (22′-pyridinedicarboxylic acid).

The amount of this acid contained is 0.32% to that of the viscous substance of fresh natto, and 0.77% in the case of older one. The growth of Bacillus natto is depressed in the presence of 0.4∼0.6%, and completely supressed when the percentage of this acid runs up as high as 1.2∼1.5%. An attempt has been made by the writer to produce natto with a high percentage of the vicious substance by the addition to the boiled beans of proteine, carbohydrate and some inorganic substances in various proportions, but it was fruitless. The causes of failure in this case seems to be twofold: in the first place, dipicolinic acid, ammonia and other substances poisonous to Bacillus natto are chemically produced in the course of fermentation, and in the second place, the very production of vicious substance had an injurious effect on the growth of the bacillus.

Dipicolinic acid has also an injurious effect on various other microorganisms, its action being decidedly so in the case of the bacilli the writer has experimented with, than in the case of Bacillus natto. In fact, Bac. subtilis, Sakè yeast, Zygosaccharomyces major, Willia anomala, Penicillium glaucum and others were found to be quite unable to multiply in the presence of dipicolinic acid in the amount existing in the ordinary natto. Hence it must be concluded that the production of this acid plays an antiseptic part on natto to considerable degree.

On the New Measuring Apparatus of Hydrogen Value of Fats and Oils. (pp. 395∼400): Tutomu MARUYAMA. (Chemical Research Institute of Kyoto University.)

Studies on the Castor-Bean Lipase. IX.—On the enzymic oil-synthesis and its activating substance. (pp. 401∼405): By Etsuo TAKAMIYA.

(Biochemical Laboratory, Department of Agriculture, Kyushu Imperial University, Fukuoka, Japan.)

The existence of a new substance (A. S) in castor-seeds which has an ability of activation or retardation on the enzymic oil-hydrolysis corresponding to changing its form by oxidation was reported in the previous paper.

Now a new fact was also discovered that such A. S substance having no effect on an enzymic oil-synthesis becomes to have an ability of activation on it by an air-contact treatment and the stronger the air-contact, the greater a tendency of the activation on oil-synthesis becomes. Dioxystearic acid of which a large quantity exists in the A. S substance has no effect on oil-synthesis and A. S substance oxidised by means of H_2O_2 retards the synthetic action of lipase. The enzyme preparation purified by author's method losses greately its synthetic action by the air-contact treatment.

Although it is yet unknown what change in the A. S substance occurs by the air-contact treatment, not a general oxidation but some intermolecular change may probably occurs. A mechanism of the A. S substance on its activating functions to enzymic hydrolysis and synthesis corresponding to changing its form by the air-contact treatment may possibly be due to changing an oxidation-reduction potential of the enzymic reaction-medium. In other words, there may exist different optimum oxidation-reduction potentials respectively for enzymic hydrolysis and synthesis. And ther may probably not exist different special enzymes respectively for enzymic hydrolytic and synthetic action, but one and the same enzyme due only to its oxidation-reduction potential of the enzymic reaction-medium may display the hydrolytic or synthetic action.

Enzyme preparation	Substance added	Degree of synthesis(%)		Degree of hydrolysis(%)	
		control	test	control	test
Y(CO_2)	A.S (CO_2) original	8.2	7.1	37.0	46.6
Y(CO_2)	A.S (CO_2) vacuum dry	8.5	12.9	36.0	41.2
Y(CO_2)	A.S (CO_2) aeration	3.2	13.8	40.6	42.1
Y(CO_2)	A.S (CO_2) H_2O_2	5.2	2.8	41.4	19.3
Y	A.S vacuum dry	24.4	32.9	14.8	12.9
Y	A.S dioxystearic acid	11.7	12.9	18.5	18.2

Some Properties of Benzyl-cellulose. (pp. 406~407): By Sojiro KAWASE and Yoshijiro KIHARA. (Agr. Chemical Laboratory of the University of Tokyo, Japan.)

Biochemical Studies of the Locust. (1). (pp. 408~411): By Chikafumi ICHIKAWA. (The Soma Agricultural School.)

Biochemical Studies of the Locust. (2). (pp. 412~414): By Chikafumi ICHIKAWA. (The Soma Agricultural School.)

Comparison of Nutritive Value of Fodder Proteins I. (pp. 415~420): By Hisayoshi IWATA. (Imperial College of Agriculture and Forestry, Morioka, Japan.)

The nutritive value of proteins in protein rich fodders, such as sardine cake, soy bean cake, extracted soy bean, rape seed cake, sesam cake, wheat bran and rice bran, and their mixtures, were experimentally compared by feeding young albino rats. For the purpose of finding the correlation between the nutritive value of protein and the amino acids composition, the latter was also examined.
